Supporting Information (SI Appendix)

Inflammation induces dermal Vγ4+ γδT17 memory-like cells that travel to distant skin and accelerate secondary IL-17-driven responses

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Materials and Methods

Mice.

WT C57BL/6 and *Sox13*$^{mut/mut}$ $CD45.1^+$ congenic mice were from the National Cancer Institute (NCI) or from Charles River Laboratories (CR). WT $CD45.1^+$, $Thy1.1^+$ congenic, and Nur77-GFP (1) mice were from Jackson laboratories and bred in our colony. *Ccr2*$^{-/-}$ mice were provided by Q. Tang (2). L*tβr*$^{-/-}$ mice were provided by K. Pfeffer (3). *Il1r1*$^{-/-}$ mice were provided by A. Sil (4). Animals were housed in a specific pathogen–free environment in the Laboratory Animal Research Center at UCSF.

Tissue preparation.

Ears were split into dorsal and ventral halves and digested for 120 min at 37 °C, with

rotation, in DMEM containing penicillin-streptomycin, HEPES buffer, 85 μg/ml Liberase TM (Roche Applied Science), 100 μg/ml DNAseI (Sigma), 0.5 mg/ml hyaluronidase (Sigma) and 2% FCS, as described (5). Digestion enzymes were quenched by the addition of 5 mM EDTA and 1% serum. In some experiments, split ear halves were digested in HBSS plus 1 Wünsch U/ml Liberase TL (Roche) for 45 min at 37°C with constant agitation (6). Ear digests were disaggregated by passage through a 70-μm or 100-μm nylon sieve (BD Bioscience).

Flow cytometry.
Cell suspensions were stained as described (5) with the following antibodies: anti-Vγ4 (UC3-10A6), anti-CD45.2 (104; from BD Biosciences or Biolegend); anti-Vδ4 (GL2), anti-γδTCR (GL3), anti- anti-CD11b (Mac-1), anti-Ly6G (1A8), anti-CLA (HECA-452), anti-IL-1R1 (JAMA-147), anti-CD25 (PC61), and anti-CD45.1 (A20; all from Biolegend); anti-TCRβ (H57-597; eBioscience or Biolegend); anti-IL-17A (eBio17B7; eBioscience); anti-CCR6 (140706; BD Biosciences); anti-S1PR1 and anti-CCR2 (475301, R&D Systems). S1PR1 was detected as described (7). For intracellular staining of skin cells, ear skin was digested in the presence of brefeldin A (BD Biosciences) and then stained as described (5). BrdU detection was performed according to the manufacturer's protocol (BD Pharmingen). For detection of CD62E and CD62P ligands, cells were incubated in 10 ug/mL CD62E/Fc and CD62P/Fc chimeras (R&D Systems) in HBSS supplemented with 2 mM calcium, 5% FCS, and 1 mM HEPES (8).

Mouse treatments.
Induction of psoriasis-like inflammation on ear skin was done as described (5). Mice between 8 and 12 weeks of age were treated daily for up to 7 days on each ear with 5 mg of 5% imiquimod cream (Imiquimod Cream 5%; Fougera Pharmaceuticals) or control cream (Vanicream; Pharmaceutical Specialties). For short-term sensitization assays, only the left ear was treated for the first 5 days, followed by 3 days of treatment on the right ear only. For analysis of persistence of $V\gamma4^+V\delta4^+$ γδT cells in previously inflamed skin, tissues were analyzed at different time points following daily ear skin treatment as above for 5 days. For long-term sensitization experiments, mice were control or IMQ treated (50 mg) on shaved and depilated back skin and allowed to recover for at least 1 month. For re-challenge assays, ear skin was treated with IMQ daily for 3 days at least one month after sensitization (IMQ treatment of back skin for 5 days). Where indicated, mice received 1mg/kg FTY720 (custom synthesis, SRI International) in normal saline by intraperitoneal (i.p.) injection, dosed every other day during the course of IMQ treatment. Where indicated, control-treated or back skin IMQ-sensitized mice as above received 10 mg of mannan from the yeast S. cerevisiae (M7504, Sigma–Aldrich) dissolved in 200 μL of PBS via i.p. injection, and ear skin inflammation was monitored for 5 days, at which point ear skin and LNs were harvested (9). For *in vivo* analysis of IL-1β response, control or sensitized mice as above received IL-1β (25 ng, PreproTech) by intradermal injection into L ear skin on day 0 and day 2, PBS was used as control on the right ear. On day 3, draining and non-draning CLN were harvested. Ear thickness was measured with digital calipers (Mitutoyo). For histological analysis, paraformaldehyde-fixed, paraffin-embedded ear skin sections were prepared and stained with hematoxylin and eosin by the University of California San Francisco Mouse Pathology Core.

Adoptive transfers and *in vivo* BrdU Labeling.
For assessment of homing to inflamed skin, the ear and back skin of WT CD45.2$^+$ or *Ccr2*$^{-/-}$ CD45.2$^+$ donor mice was treated with IMQ as above daily for 5 days, at which point draining (cervical, axillary, inguinal) LN cells were harvested. WT cells were labeled with CellTrace Violet (CTV, Molecular probes, according to the manufacturer's instructions), mixed with unlabeled *Ccr2*$^{-/-}$ cells and transferred by intravenous injection into CD45.1$^+$ (*Sox13*$^{mut/mut}$) mice that had been treated on ear skin with IMQ for 2-3 days (a total of 5x10^7 cells were transferred). Three to 8 hrs after transfer, ear skin of recipient mice was harvested. In some experiments, the labeling protocol was reversed, with CTV-labeled *Ccr2*$^{-/-}$ cells mixed with unlabeled WT cells. For assessment of intrinsic memory response, back skin of WT CD45.2$^+$ or Thy1.2$^+$ mice was treated as above and 5x10^7 draining LN cells transferred into congenically marked WT (CD45.1$^+$ or Thy1.1$^+$) recipients. Two-4 weeks after transfer, ear skin of recipient mice was treated with IMQ daily for 3 days. At d3, recipient mice received 2.5 mg of BrdU (Sigma–Aldrich) by i.p. administration, and tissues were harvested 30 minutes later.

Chemotaxis.
Transwell assays were performed as described (7). Draining LN cells from mice treated with IMQ for 5 days were resuspended in RPMI medium containing 10mM HEPES and 2% fatty acid free BSA and tested for transmigration across uncoated 5μm transwell filters (Corning Costar Corp.) for 3 h to medium alone or to of CCL2 (100 ng/mL, PreproTech).

***In vitro* assays.**
For analysis of the effect of TCR or cytokine signaling on Nur77 expression, LN cells from Nur77-GFP mice were incubated in plates coated with control IgG or 3 μg/mL anti-CD3ε (145-2c11, Bio-x-cell) or in the presence of IL-1β and IL-23 (10 ng/mL each, PreproTech) for 18 hrs.

Real-time PCR.
Total RNA was isolated and converted to cDNA as described (5). A StepOnePlus real-time PCR system (Applied Biosystems) with iTaq SYBR Green Supermix (Bio-Rad) and the appropriate primer pairs (Integrated DNA Technologies) were used for real-time PCR. Primers for *Il17a*, *Il17f*, *Defb3* and *Defb4,* and *Cxcl2* have been described (5). Primers for *Ccl2* were TGGCTCAGCCAGATGCAGT (forward) and TCTTTGGGACACCTGCTGCT (reverse). Primers for *Il7* were GTGCCACATTAAAGACAAAGAAG (forward) and GTTCATTATTCGGGCAATTACTATC (reverse).

Supplementary References.

1. Moran AE, et al. (2011) T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *J Exp Med* 208(6):1279–1289.

2. Boring L, et al. (1997) Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *J Clin Invest* 100(10):2552–2561.

3. Fütterer A, Mink K, Luz A, Kosco-Vilbois MH, Pfeffer K (1998) The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. *Immunity* 9(1):59–70.

4. Glaccum MB, et al. (1997) Phenotypic and functional characterization of mice that lack the type I receptor for IL-1. *J Immunol* 159(7):3364–3371.

5. Gray EE, et al. (2013) Deficiency in IL-17-committed Vγ4(+) γδ T cells in a spontaneous Sox13-mutant CD45.1(+) congenic mouse substrain provides protection from dermatitis. *Nat Immunol* 14(6):584–592.

6. Cheng LE, et al. (2015) IgE-activated basophils regulate eosinophil tissue entry by modulating endothelial function. *J Exp Med*.

7. Arnon TI, et al. (2011) GRK2-dependent S1PR1 desensitization is required for lymphocytes to overcome their attraction to blood. *Science* 333(6051):1898–1903.

8. Hirahara K, et al. (2006) The majority of human peripheral blood CD4+CD25highFoxp3+ regulatory T cells bear functional skin-homing receptors. *J Immunol* 177(7):4488–4494.

9. Khmaladze I, et al. (2014) Mannan induces ROS-regulated, IL-17A-dependent psoriasis arthritis-like disease in mice. *Proc Natl Acad Sci U S A* 111(35):E3669–3678.

Supplementary Figures

Figure S1

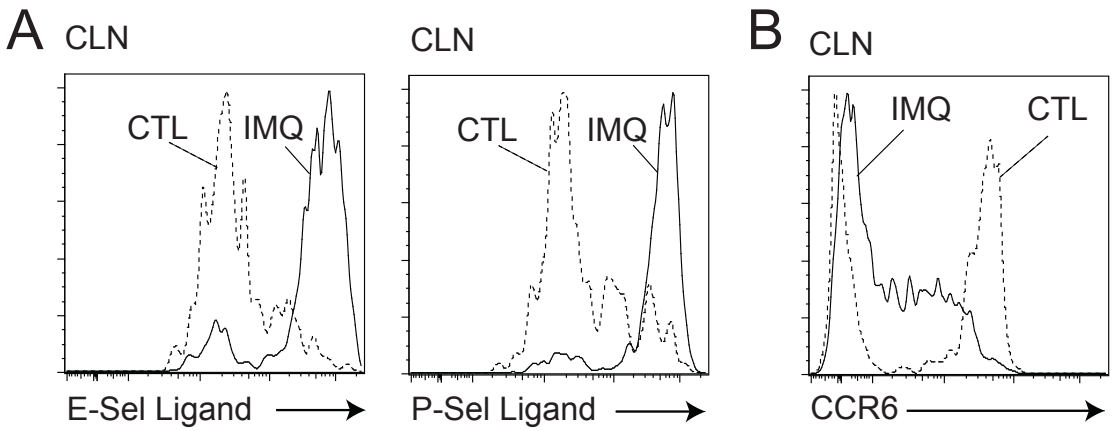

Figure S1. Expression of E- and P-selectin ligands and CCR6 by IMQ-expanded LN Vγ4$^+$Vδ4$^+$ γδT17 cells. (A) E- (left panel) and P-selectin (right panel) ligand expression on Vγ4$^+$Vδ4$^+$ γδT cells from draining CLN after treatment for 5 days with control (CTL) cream or IMQ cream on ear skin. Data are representative of two experiments. (B) CCR6 cell surface expression on Vγ4$^+$Vδ4$^+$ γδT cells from CLN of mice treated as in A. Data are representative of at least 4 experiments with at least 3 mice each.

Figure S2

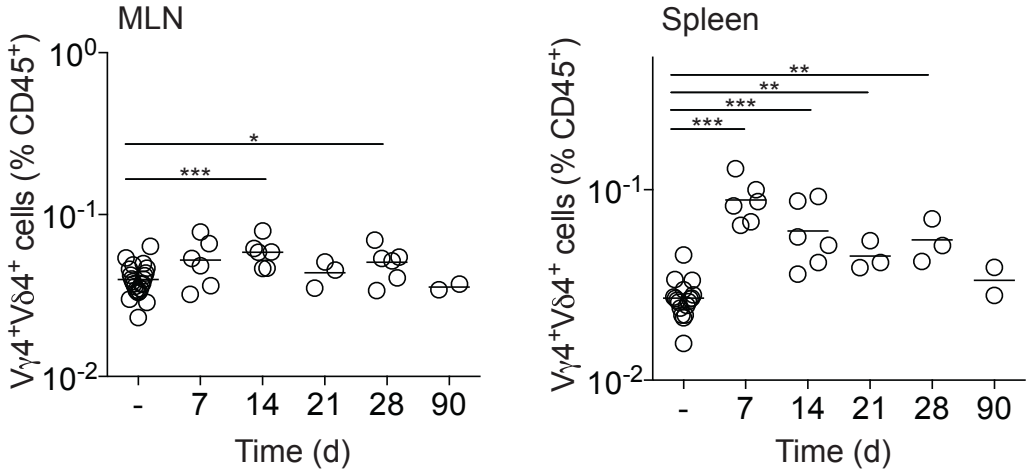

Figure S2. Expanded Vγ4$^+$Vδ4$^+$ γδT17 cells redistribute preferentially to peripheral LNs. Frequency of Vγ4$^+$Vδ4$^+$ γδT17 cells in the indicated tissues in control (-) mice or animals that were treated with IMQ on ear skin daily for 5 days (d0-d5) and harvested at the indicated times. Data are pooled from at least one experiment at each time point, with at least 2 mice in each group. **p<0.01, ***p<0.001

Figure S3

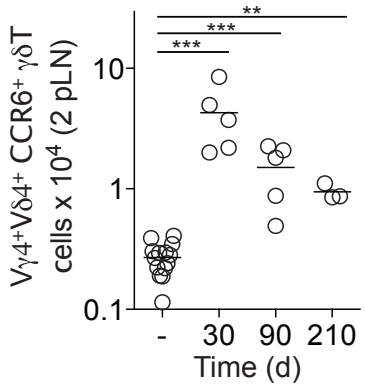

Figure S3. Previously activated Vγ4$^+$Vδ4$^+$ γδT17 cells persist in peripheral LNs. Vγ4$^+$Vδ4$^+$ CCR6$^+$ γδT cell number in ILN of mice that were treated on back skin with control cream (-) or IMQ daily for 5 days, and harvested at the indicated times. Data are pooled from at least one experiment at each time point, with at least 2 mice in each group. **p<0.01, ***p<0.001

Figure S4

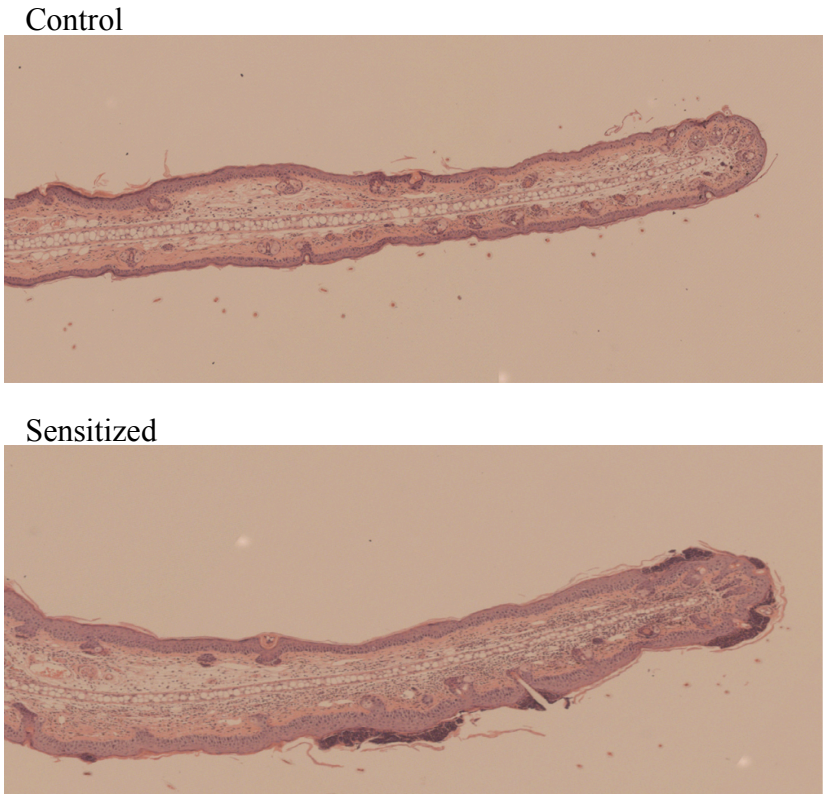

Figure S4. Accelerated psoriasis-like inflammation in previously untreated skin of IMQ-sensitized mice. Hematoxylin-and-eosin staining of murine ear skin treated with IMQ daily for 3 days from mice that had been treated with control cream (top) or IMQ-sensitized (bottom) on back skin for 5 days one month prior to ear skin treatment. Data are representative of 3 experiments with 3 mice each.

Figure S5

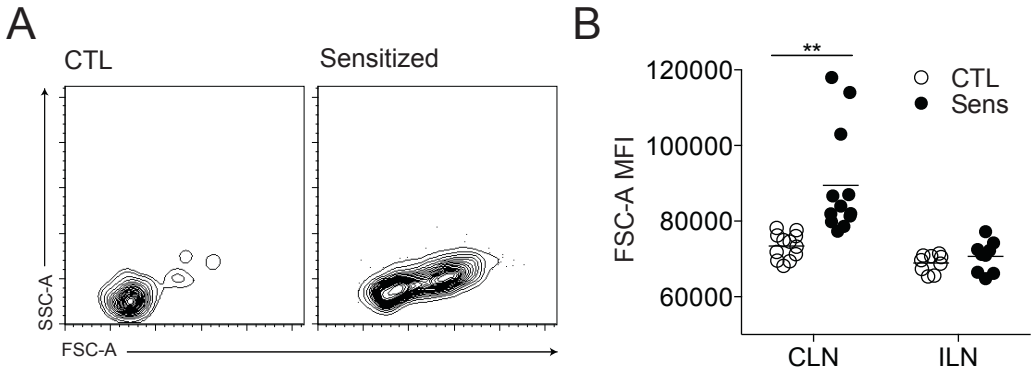

Figure S5. Increased size of Vγ4$^+$Vδ4$^+$ γδT cells in responding LNs of sensitized mice after IMQ treatment. (A) Representative plots of forward vs. side scatter of CLN Vγ4$^+$Vδ4$^+$ γδT cells in control (left) or sensitized (right) mice after 3 days of ear skin treatment with IMQ. (B) Forward scatter MFI of Vγ4$^+$Vδ4$^+$ γδT cells from mice treated as in A, from draining CLNs or non-draining ILNs. Data are pooled from 4 (CLN) or 3 (ILN) experiments with 3 mice in each group. **p<0.01

Figure S6

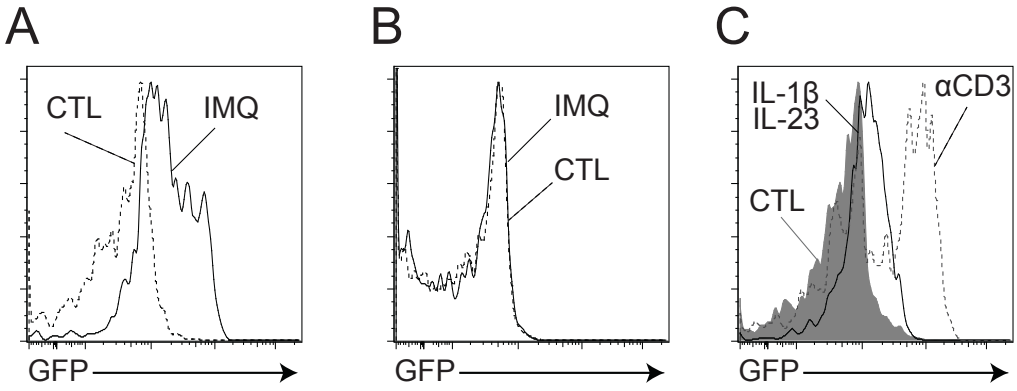

Figure S6. Nur77 expression in activated Vγ4$^+$ γδT17 cells *in vivo* an *in vitro*. GFP expression in Vγ4$^+$ CCR6$^+$ cells from ear skin (A) or draining LN (B) of Nur77-GFP mice treated with control (CTL) cream or IMQ for 2 days. Data are representative of 2 experiments and 3 mice. (C) GFP expression in Vγ4$^+$ CCR6$^+$ cells from LN of Nur77-GFP mice cultured for 18 hrs in plates coated with control IgG (CTL), anti-CD3ε (3 μg/mL), or in the presence of IL-1β and IL-23 (10 ng/mL). Data are representative of two experiments in duplicate.

Figure S7

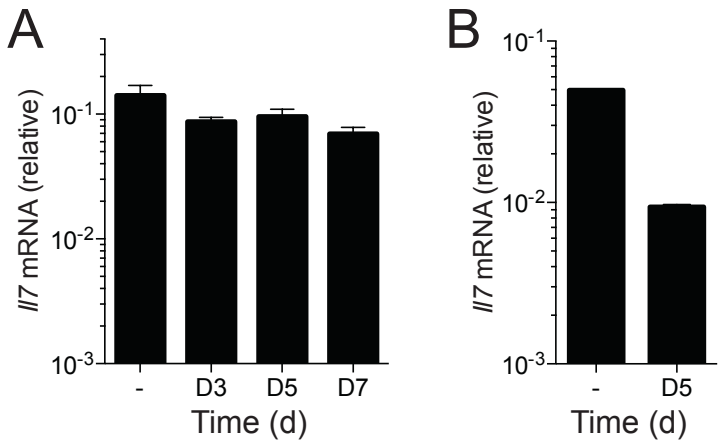

Figure S7. IL-7 expression in IMQ treated mice. RT-PCR analysis of *Il7* mRNA in draining LN (A) or ear skin (B) from control (-) or mice treated with IMQ for the indicated time. Data are pooled from at least two experiments with 2 mice of each type (A), or two experiments with one mouse each (B). Bars indicate mean +/- SEM.

Figure S8

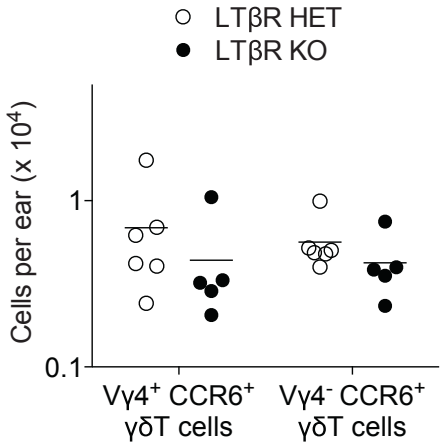

Figure S8. Number of dermal γδT17 cells in skin of mice that lack lymph nodes is similar to control mice. Cell numbers in ear skin of *Ltbr*$^{-/-}$ mice, which lack lymph nodes, and *Ltbr*$^{+/-}$ control mice. Data are pooled from 3 experiments with at least one mouse of each type.